

Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D

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Loss of neural input to skeletal muscle fibres induces atrophy and degeneration with evidence of mitochondria-mediated cell death. However, the effect of denervation on the permeability transition pore (PTP), a mitochondrial protein complex implicated in cell death, is uncertain. In the present study, the impact of 21 days of denervation on the sensitivity of the PTP to Ca^{2+} -induced opening was studied in isolated muscle mitochondria. Muscle denervation increased the sensitivity to Ca^{2+} -induced opening of the PTP, as indicated by a significant decrease in calcium retention capacity (CRC: 111 ± 12 versus 475 ± 33 nmol (mg protein)⁻¹ for denervated and sham, respectively). This phenomenon was partly attributable to *in vivo* mitochondrial and whole muscle Ca^{2+} overload. Cyclosporin A, which inhibits PTP opening by binding to cyclophilin D (CypD), was significantly more potent in mitochondria from denervated muscle and restored CRC to the level observed in mitochondria from sham-operated muscles. In contrast, the CypD independent inhibitor trifluoperazine was equally effective at inhibiting PTP opening in sham and denervated animals and did not correct the difference in CRC between groups. This phenomenon was associated with a significant increase in the content of the PTP regulating protein CypD relative to several mitochondrial marker proteins. Together, these results indicate that Ca^{2+} overload *in vivo* and an altered expression of CypD could predispose mitochondria to permeability transition in denervated muscles.

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Several progressive neuromuscular diseases are associated with severe atrophy and loss of muscle fibres resulting in muscle wasting as the main clinical presentation. There is growing evidence that activation of cell death through apoptosis, and in a lesser manner necrosis, is involved in the loss of muscle fibres (Lu *et al.* 1997; Borisov & Carlson, 2000; Tews, 2002; Siu & Alway, 2005). In addition, apoptosis also occurs in localized segments of the remaining myofibres and may play a role in atrophic remodelling (Allen *et al.* 1997, 1999; Leeuwenburgh *et al.* 2005). These phenomena have been described in denervation disorders (Tews & Goebel, 1997b; Yoshimura & Harii, 1999; Borisov & Carlson, 2000; Siu & Alway, 2005), muscular dystrophy (Sandri *et al.* 1995, 2001), inactivity (Leeuwenburgh *et al.* 2005; Siu *et al.* 2005a) and ageing-induced sarcopenia (Leeuwenburgh *et al.* 2005; Siu *et al.* 2005a,b).

Although the mechanisms responsible for the activation of cell death pathways in muscle are not well defined,

there is evidence that at least in denervation atrophy, mitochondria may play a role. Indeed, several studies have reported an increase in the bax/bcl-2 ratio in various denervation disorders (Tews & Goebel, 1996, 1997a,b; Tews, 2002; Siu & Alway, 2005), which was suggested to induce permeation pathways through mitochondrial membranes. In addition, recent studies have shown that 2 weeks of denervation in rat muscle leads to the release of several mitochondrial pro-apoptotic proteins (e.g. cytochrome *c*, AIF, smac/Diablo) and to the activation of downstream events including the activation of caspase 9 and 3 and DNA fragmentation (Siu & Alway, 2005).

One of the well-described mechanisms of membrane permeation in mitochondria involves the opening of the permeability transition pore (PTP) (see Zoratti & Szabo, 1995 for extensive review). The PTP is a high conductance non-specific pore presumably formed by a supramolecular complex spanning the double membrane system of the mitochondria mainly at contact sites. Although it is

increasingly recognized that the molecular composition of the PTP is probably variable (He & Lemasters, 2002; Kim *et al.* 2003; Zoratti *et al.* 2005), the prevailing hypothesis is that the adenylate translocator (ANT), the porin pore (VDAC) and the regulatory matrix protein cyclophilin-D (CypD) are the major proteins forming the complex (Crompton, 1999; Halestrap *et al.* 2000). Opening of the PTP results in matrix swelling, loss of membrane potential ($\Delta\Psi$), uncoupling of oxidative phosphorylation, and ATP hydrolysis and has also been shown to cause the release of several mitochondrial pro-apoptotic proteins including cytochrome *c*, AIF, Smac/Diablo, endonuclease G and Omi/HtrA2 (Di Lisa & Bernardi, 1998; Bernardi, 1999; Bernardi *et al.* 1999; Crompton, 1999; Suleiman *et al.* 2001). Although the regulation of PTP gating varies depending on the tissue studied, accumulation of Ca^{2+} in the matrix is the obligatory and most important trigger for its opening (Zoratti & Szabo, 1995; Bernardi, 1999). Several factors, including the type of substrate used, membrane potential, redox state of pyridine nucleotides, reactive oxygen species, adenylate concentration and pH can, however, act as coregulators by affecting the sensitivity of the pore to Ca^{2+} (Zoratti & Szabo, 1995; Bernardi, 1999).

There is currently little data available on the PTP in skeletal muscle. Opening of the PTP was shown to mediate the myotoxic effect of the anaesthetic bupivacaine, which could be prevented by inhibiting the pore with cyclosporin-A (CsA) (Irwin *et al.* 2002). More recently, mitochondrial dysfunction caused by an increased vulnerability to PTP opening was observed in mice harbouring a knockout of the collagen VI gene, which is involved in Bethlem myopathy and Ullrich congenital muscular dystrophy (Irwin *et al.* 2003). However, whether the vulnerability of mitochondria to PTP opening is altered in denervated muscle remains largely unknown. In the present study, surgical denervation was used to determine whether loss of neural input and/or its functional sequelae alters the sensitivity of isolated mitochondria to PTP opening and if this could be associated with changes in some factors favouring permeability transition.

Methods

Muscle denervation procedure

Female Sprague-Dawley rats (Charles River, Saint-Constant, Quebec, Canada), weighing 225–250 g at the beginning of the experiment, were housed in an environmentally controlled room (23°C, 12 : 12 h light–dark cycle) and provided water and food *ad libitum*. Animals were randomly assigned to one of two groups: muscle denervation or sham-operated control where $n = 24$ animals per group. Following anaesthesia (ketamine–xylazine: 61.5/7.7 mg kg⁻¹, i.p.) muscle denervation was performed bilaterally by extracting

a 10 mm segment of the sciatic nerve through an incision in the mid-posterolateral area of the thigh. The incision was closed with silk sutures and the animals were administered buprenorphine (0.05 mg kg⁻¹, i.p.) as postoperative analgesia. The incision site was washed with antibacterial solution to prevent infection. Daily inspection of the animals' hindlimbs was performed, verifying for absence of the toe-spread reflex. Body mass was evaluated every 48 h as an indication of tolerance to the denervation procedure. Any animal demonstrating signs of distress or automutilation of the distal ends of the feet and/or toes was immediately excluded from the experimental protocol. All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

Materials

All chemicals were purchased from Sigma (St Louis, MO, USA), with the exception of Cyclosporin A (Tocris, Ellisville, MO, USA), and Calcium Green-5N (Molecular Probes, Eugene, OR, USA).

Mitochondrial isolation procedure

Isolation of mitochondria was performed as previously described (Fontaine *et al.* 1998) with minor modifications. Twenty-one days following surgical denervation, animals were anaesthetized (ketamine–xylazine: 61.5/7.7 mg kg⁻¹, i.p.) and the plantar group of muscles (soleus, plantaris, gastrocnemius) was dissected from the surrounding connective tissue, rapidly removed, trimmed clean of visible connective tissue, weighed and placed in 10 ml of ice-cold mitochondrial isolation buffer (mm: 150 sucrose, 75 KCl, 50 Tris base, 1 KH₂PO₄, 5 MgCl₂, 1 EGTA, 0.2% BSA, pH 7.4). Animals were subsequently killed by lethal overdose of ketamine–xylazine. All steps were performed at 4°C. Muscles were minced with scissors, incubated for 1 min with Nagarse protease (0.2 mg ml⁻¹) and homogenized using a motor-driven Teflon pestle homogenizer. The homogenate volume was completed to 40 ml with cold isolation buffer and centrifuged at 700 g for 10 min. The supernatant was decanted and centrifuged at 10 000 g for 10 min. The pellet was resuspended in 40 ml of suspension buffer (mm: 250 sucrose, 10 Tris base, 0.1 EGTA, pH 7.4) and centrifuged at 8000 g for 10 min. The final mitochondrial pellet was resuspended in 0.5 ml of suspension buffer and protein concentrations were determined by the bicinchoninic acid method.

Respirometry and enzyme activity

Mitochondrial oxygen consumption was measured polarographically at 25°C, using Clark-type electrodes

(Oxygraph, Hansatech Instruments, Kings Lynn, UK). Experiments were started with the addition of 0.3 mg mitochondria in 1 ml of respiration buffer (mM: 125 KCl, 10 P_i-Tris, 0.05 EGTA, 10 Mops, 2.5 MgCl₂) supplemented with glutamate–malate (5 : 1 mM). The medium was then supplemented with 0.25 mM ADP to measure maximal rate of oxidative phosphorylation (V_{ADP}).

For the measurement of the activity of the respiratory chain complex IV cytochrome oxidase (COX), aliquots of the mitochondrial suspension were treated with 0.1% Triton X-100 for 60 min on ice. Following centrifugation for 5 min at 10 000 g, COX activity in the supernatant was determined spectrophotometrically as previously described (Burelle & Hochachka, 2002) and reported in milliunits per milligram protein.

Ca²⁺ challenge

Mitochondria (0.15 mg ml⁻¹) were incubated at 25°C in 1.5 ml of buffer (mM: 250 sucrose, 10 Mops, 0.05 EGTA, 10 P_i-Tris, pH 7.3) containing glutamate–malate (5 : 2.5 mM) or succinate–rotenone (5 mM–1 µM). All substrates were free acids buffered to pH 7.3 with Tris. Changes in extra-mitochondrial calcium concentration were monitored fluorimetrically (Hitachi, F4500 or Ocean Optics SD2000 spectrofluorometer) using Calcium Green-5N (1 µM, excitation–emission: 505–535 nm) as described by Ichas *et al.* (1997). Residual calcium concentration was adjusted to the same level at the beginning of every experiment by adding a small amount of EGTA. Unless stated otherwise, calcium pulses (83 nmol (mg prot.)⁻¹) were then added at 2 min intervals until a Ca²⁺-induced mitochondrial Ca²⁺ release was observed. We have previously shown that this Ca²⁺ release is invariably accompanied by loss of $\Delta\Psi$ and high amplitude swelling of the mitochondrial matrix (Marcil *et al.* 2005). Calcium retention capacity (CRC) was taken as the total amount of Ca²⁺ accumulated by mitochondria prior to the Ca²⁺ pulse triggering Ca²⁺ release. This value represents a reliable index of the threshold [Ca²⁺] required to open the PTP in the whole mitochondrial population studied.

Endogenous Ca²⁺ content

Muscles were sampled from one hindlimb and freeze-clamped in liquid N₂ for determination of whole muscle Ca²⁺ content. Muscles from the contralateral limb were used for the isolation of mitochondria as described above except that all buffers were free of EGTA in order to avoid chelating Ca²⁺. Ground muscle samples and isolated mitochondrial pellets were diluted in 0.6 N HCl (1/10 w/v), homogenized with a polytron (2 × 10 s at a setting of 3) and sonicated (2 × 10 s at

40% of maximal power output). Following 30 min of incubation in boiling water, samples were centrifuged 5 min at 10 000 g and the supernatant was recovered. Ca²⁺ content in the supernatant was determined spectrophotometrically (VERSAmix, Molecular Devices) using an O-Cresolphthalein Complexone assay according to the manufacturer's instructions (TECO Diagnostics, Anaheim, CA, USA). Results were expressed in nanomoles Ca²⁺ per milligram protein.

Immunoblot analysis

The protein expression of CypD, ANT and VDAC was determined in the isolated mitochondrial fraction. Samples were prepared for SDS-PAGE by dilutions with reducing sample buffer followed by a 10 min immersion in near-boiling water. Twenty micrograms of protein were loaded in each lane and resolved on 12% polyacrylamide mini-gels at room temperature. The gels were transferred to a PVDF membrane (Millipore). Equal sample loading was confirmed by Ponceau S stain (Sigma-Aldrich). The membrane was fixed for 10 min with 0.05% glutaraldehyde in phosphate-buffered saline with 0.1% Tween 20 (PBS-T) then blocked in 5% non-fat milk (CypD, VDAC) or 5% BSA (ANT) in PBS-T at room temperature for 90 min, and incubated overnight at 4°C with the following primary antibodies diluted in PBS-T with 5% BSA: anti-CypD (1 : 2000 dilution, Affinity Bioreagents, Golden, CO, USA), anti-VDAC (1 : 2000 dilution, Alexis Biochemicals, San Diego, CA, USA) and anti-ANT (1 : 2000 dilution, Calbiochem San Diego, CA). Membranes were then incubated for 90 min at room temperature in secondary antibody solution (1 : 50000 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Bands were visualized by enhanced chemiluminescence (Amersham) with film exposure times ranging from 5 to 45 min. Films were scanned and bands quantified by ImagePro software.

Statistical analysis

Data were analysed using ANOVA followed by Fisher's *post hoc* test. Where appropriate (i.e. in Ca²⁺ challenge experiments), Bonferroni's correction was applied to the *P*-value obtained to correct for multiple comparisons. A corrected *P*-value < 0.05 was considered significant.

Results

Morphometric parameters and mitochondrial protein isolation yield

Denervation for 21 days caused an atrophy of the hindlimb muscles as evidenced by a significant reduction

Table 1. Morphometric data and mitochondrial yield in muscle from sham and denervated animals

	Sham	Denervated
Body weight (g)	262.3 ± 4.3	262.0 ± 3.7
Hindlimb muscle weight (mg)	1679 ± 27	649 ± 15*
Muscle mass to body mass ratio (mg g ⁻¹)	6.42 ± 0.009	2.49 ± 0.05*
Mitochondrial isolation yield (mg prot. g ⁻¹)	3.926 ± 0.286	3.207 ± 0.388
Mitochondrial protein yield (mg)	6.610 ± 0.449	3.332 ± 0.322*

*Significantly different from sham, $P < 0.05$

in absolute muscle mass and muscle mass to body mass ratio (Table 1). The amount of protein recovered in the mitochondrial fraction was not significantly different between the two experimental groups. However, because of muscle atrophy, the total amount recovered per gram of muscle was significantly lower in denervated compared to sham-operated animals.

Respiratory parameters

Figure 1 shows the results of respirometry experiments aimed to determine oxidative capacity. In mitochondria from denervated muscles, basal ADP restricted (V_0) and maximal rate of ADP-stimulated (V_{ADP}) respiration were significantly decreased relative to sham when expressed per milligram of protein. This reduction of respiratory activity was similar in magnitude to that of the respiratory chain marker enzyme COX, which was 1.8-fold lower in denervated compared to sham-operated animals. Consequently, when respiration rates were normalized per unit of COX, V_0 and V_{ADP} were similar in the two experimental groups.

Vulnerability to Ca²⁺-induced PTP opening

Figure 2 shows representative Ca²⁺ uptake traces in mitochondria from sham and denervated muscles submitted to progressive Ca²⁺ loading. When energized with the complex II substrate succinate, mitochondria from sham muscle were able to accumulate 475 ± 33 nmol Ca²⁺ (mg protein)⁻¹ before Ca²⁺ was released as a result of PTP opening. In contrast, under the same conditions 4.3-fold less Ca²⁺ was required to trigger pore opening in mitochondria from denervated muscle (111 ± 12 nmol Ca²⁺ (mg protein)⁻¹) and the Ca²⁺ uptake kinetics were substantially slower compared to sham (time to 50% uptake: 48 ± 13 versus 14 ± 1 s, $P < 0.05$).

In energized mitochondria, the regulation of PTP opening is known to vary according to the type of substrate oxidized, with substrates feeding complex I acting as sensitizers compared to substrates for complex II (Fontaine *et al.* 1998; Bernardi, 1999; Leverve & Fontaine, 2001; Marcil *et al.* 2005). Therefore, experiments

were also performed in the presence of the complex I donors glutamate–malate (Fig. 2). In line with previous results, CRC in the presence of glutamate–malate was significantly lower than in the presence of the complex II substrate succinate in the two experimental groups. However, CRC was still reduced in denervated compared to sham-operated animals.

In order to determine whether changes in CRC could be due to changes in Ca²⁺ present in the mitochondrial matrix at baseline, endogenous Ca²⁺ levels were determined in the mitochondria and whole muscle. Figure 3 indicates that mitochondria from denervated muscles had a greater Ca²⁺ content compared to sham, which was accompanied by a substantial muscle Ca²⁺ overload.

As indicated by the lower (~2-fold) content of both inner (COX, ANT) and outer membrane (VDAC) markers (Figs 1 and 5), denervation appeared to reduce the number of mitochondria present in the mitochondrial fraction. Therefore, CRC was also determined with a fixed amount of COX (Fig. 4). Under these conditions, the difference in CRC observed between the two experimental groups was significantly less (2.4-fold) than the results obtained with a fixed amount of protein (4.3-fold). In addition, if endogenous Ca²⁺ present in mitochondria at baseline was taken into account, the difference in CRC between sham and denervated muscles decreased further to 1.5-fold, but remained significant.

Figure 4 also shows the results of experiments performed in the presence of inhibitors of the PTP. Incubation in the presence of $1 \mu\text{M}$ CsA, which inhibits pore opening by binding to the putative regulatory protein CypD (Davidson & Halestrap, 1990; Baines *et al.* 2005; Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005), resulted in a 1.6 ± 0.1 -fold increase in CRC in mitochondria from sham muscles. In contrast, in the denervated group the same treatment had a significantly more potent effect and increased CRC by 2.4 ± 0.2 -fold. As a consequence, CsA was able to abolish the difference in CRC observed between the two experimental groups. In order to determine if this phenomenon was unique to CsA, experiments were also performed in the presence of trifluoperazine, which inhibits PTP opening through a CypD-independent mechanism (Bernardi *et al.* 1993). Incubation with trifluoperazine increased CRC in both

experimental groups but did not correct for the difference in CRC observed between sham and denervated at baseline in the absence of inhibitors.

Immunoblot analysis of the mitochondrial fraction

Since the increased potency to pharmacological PTP inhibition in the denervated group appeared to be specific to CsA the mitochondrial content of CypD, the putative receptor for CsA (Davidson & Halestrap, 1990; Baines *et al.* 2005; Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005) was determined (Fig. 5). No significant difference in the absolute content of CypD was observed between groups. However similar to what was observed with COX activity (Fig. 1) the content of VDAC and ANT, two abundant mitochondrial proteins was significantly lower in the mitochondrial fraction of denervated compared to sham-operated muscles. Therefore, following denervation, CypD appeared to be over expressed by 2- to 3-fold relative to each protein marker evaluated (Fig. 5).

Discussion

Recent studies have shown that mitochondria, through their capacity to signal cell death, may be involved in the atrophic remodelling of muscles following denervation (Tews & Goebel, 1996, 1997*b,a*, Tews, 2002; Siu & Alway, 2005) but the mechanisms remain largely undefined. In the present study we show that mitochondria isolated from denervated muscles display some respiratory dysfunction and a greater susceptibility to opening of the PTP, one of the mechanisms involved in mitochondria-mediated cell death. This phenomenon is due in part to the existence *in vivo* of a muscle and mitochondrial Ca^{2+} overload, which favours permeability transition. In addition, we provide evidence that mitochondria from denervated muscle have an increased CypD content relative to several marker proteins and display a selective increase in the sensitivity to CsA, suggesting that CypD further facilitates pore opening in the denervated state.

Effect of denervation on respiratory capacity

Similar to what has been previously reported (Joffe *et al.* 1981*a*), we observed that mitochondria isolated from denervated muscle display a reduction in V_0 and V_{ADP} per milligram of protein, which would indicate that denervation caused a significant reduction in oxidative capacity. However, the amplitude of this reduction should be interpreted with caution. Indeed, while milligrams of protein is generally a good denominator to express respiratory parameters because the majority of mitochondrial proteins consist of respiratory chain

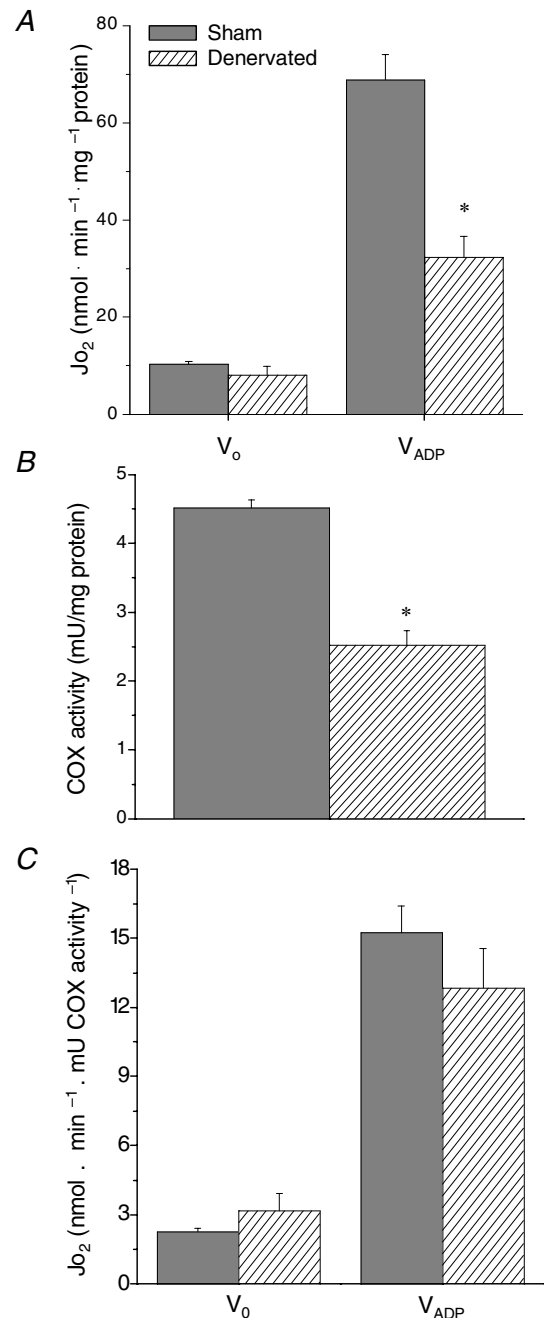


Figure 1. Respiratory function in mitochondria from sham and denervated animals

A, basal ADP-restricted (V_0), and maximal rate of oxidative phosphorylation following addition of 1 mM ADP (V_{ADP}) expressed per mg of protein. B, the activity of the terminal respiratory chain enzyme COX in the mitochondrial fraction of sham-operated and denervated animals. C, V_0 and V_{ADP} expressed per unit of COX present in the mitochondrial fraction of both experimental groups. For respirometry experiments mitochondria were energized with glutamate–malate (5, 2.5 mM). Data are presented as means \pm S.E.M., for $n = 6$ and $n = 5$ in the denervated and sham groups, respectively. *Significantly different from sham ($P < 0.05$).

enzymes, in some cases its use can introduce distortions in the results (Leary *et al.* 2003). For instance, in the present study denervation caused minimal changes in the protein concentration of the mitochondrial fraction (Table 1), whereas COX and other mitochondrial marker proteins were significantly reduced. Because the relationship between total protein and specific mitochondrial markers

was altered by denervation, respiration was also expressed per unit of COX (Leary *et al.* 2003). When expressed this way, the effect of denervation on oxidative capacity was much less pronounced, which suggests that the negative impact of denervation on the metabolic potential of mitochondria could be less important than previously thought (Joffe *et al.* 1981a).

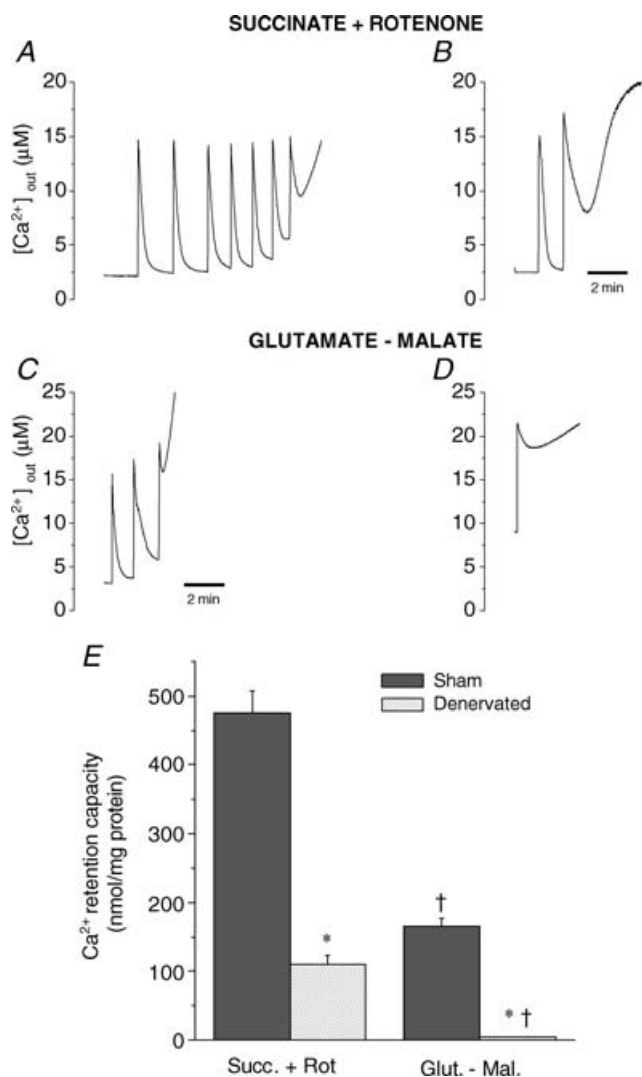


Figure 2. Response to Ca^{2+} challenge in mitochondria from sham and denervated animals

Typical Calcium Green 5N tracing of muscle mitochondria (0.15 mg ml^{-1}) energized with succinate–rotenone (5 mM , $1 \mu\text{M}$) and glutamate–malate (5 , 2.5 mM) in sham operated (A and C) and denervated animals (B and D). Tracings show progressive Ca^{2+} accumulation followed by release of accumulated Ca^{2+} secondary to PTP opening. Each spike indicates the addition of a calcium pulse of 83 nmol mg^{-1} . E, average Ca^{2+} retention capacity of mitochondria in the various experimental conditions. Means \pm s.e.m. are presented for $n = 8$ and 9 in the sham and denervated groups, respectively.

*Significantly different from sham ($P < 0.05$), †Significantly different from Succinate + Rotenone within the same experimental group ($P < 0.05$).

Effect of denervation on the susceptibility to Ca^{2+} -induced PTP opening

Previous studies in rats denervated for 21 days have reported that respiratory uncoupling occurred prematurely in mitochondria when incubated in the presence of Ca^{2+} , although the mechanisms were not investigated (Joffe *et al.* 1981b,c). The results we obtained in the present study clearly establish a role for the PTP in Ca^{2+} -induced dysfunction. Indeed, CRC was substantially lower in denervated compared to sham-operated muscles when expressed per milligram of protein or per unit of

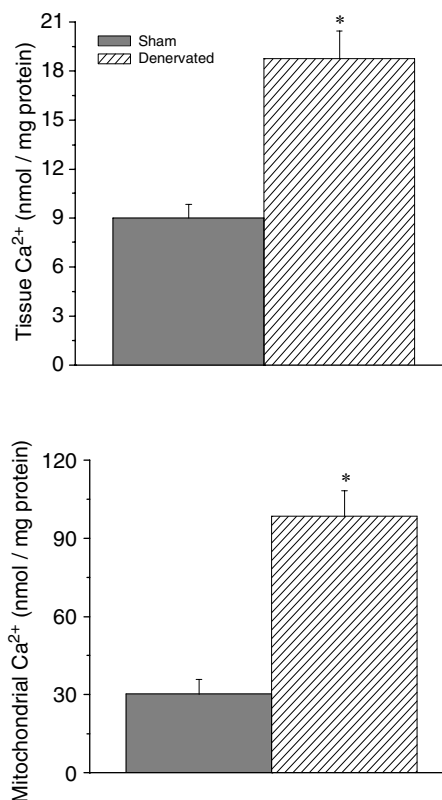


Figure 3. Effect of denervation on endogenous mitochondrial and whole muscle Ca^{2+} content in sham and denervated animals

Endogenous Ca^{2+} content measured in the whole muscle homogenate and the mitochondrial fraction of sham and denervated animals. Data are presented as means \pm s.e.m., for $n = 8$ and $n = 9$ in the denervated and sham groups, respectively. *Significantly different from sham ($P < 0.05$).

COX and the CRC displayed sensitivity to classic PTP inhibitors, particularly CsA.

Ca^{2+} -induced permeability transition is known to be modulated by a variety of physiological effectors, including the flow of electrons through complex I, which increases the sensitivity of the PTP to Ca^{2+} -induced opening (Fontaine *et al.* 1998; Bernardi, 1999; Leverve & Fontaine, 2001; Marcil *et al.* 2005). However, in the present study, bypassing complex I using succinate did not abolish the difference in CRC between both experimental groups, suggesting that the greater sensitivity of mitochondria to pore opening in denervated muscle was not related to a mechanism involving electron flow through complex I.

In accordance with a previous study (Joffe *et al.* 1981c), we observed a significant elevation in the endogenous level of Ca^{2+} in mitochondria from denervated muscles. This phenomenon has been reported in other myopathies (Joffe *et al.* 1981c; Marchand *et al.* 2001) and is probably due to a progressive increase in intramuscular Ca^{2+} levels (Joffe *et al.* 1981c and Fig. 3) which appears to occur as a result of an increase in the leakiness of the sarcolemma and the abnormal morphology and functioning of the sarcoplasmic reticulum (Joffe *et al.* 1981c; Takekura *et al.* 2003). Since the accumulation of Ca^{2+} in the matrix is the main trigger for PTP opening (Zoratti & Szabo, 1995) this increase in endogenous Ca^{2+} clearly contributed to the increased mitochondrial vulnerability to pore opening in the denervated group. However, the fact that CRC still

remained 1.5-fold lower in the denervated group after taking endogenous Ca^{2+} into account indicated that other factors further predisposed mitochondria to permeability transition.

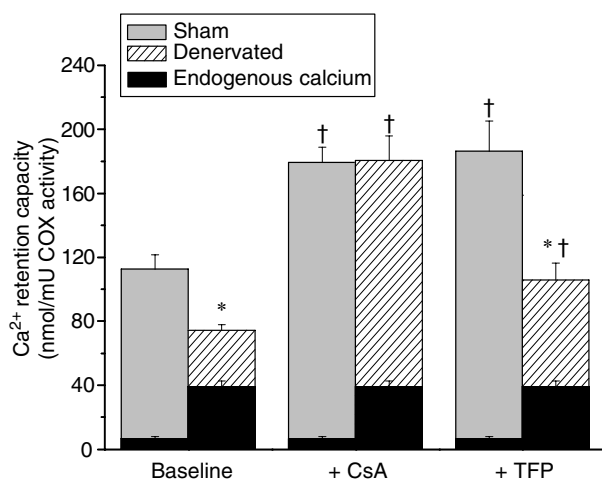


Figure 4. Total Ca^{2+} retention capacity and effect of PTP inhibitors in mitochondria from sham and denervated animals
Total Ca^{2+} retention capacity in absence and presence of the PTP inhibitors cyclosporin-A ($1 \mu\text{M}$) and trifluoperazine ($10 \mu\text{M}$). Values are expressed as the sum of endogenous Ca^{2+} (filled bars) and of Ca^{2+} added during the *in vitro* challenge and are expressed per unit of the marker enzyme cytochrome oxidase (COX). Means \pm S.E.M. are presented for $n = 8$ and 9 in the sham and denervated groups, respectively. *Significantly different from sham ($P < 0.05$), †Significantly different from baseline ($P < 0.05$).

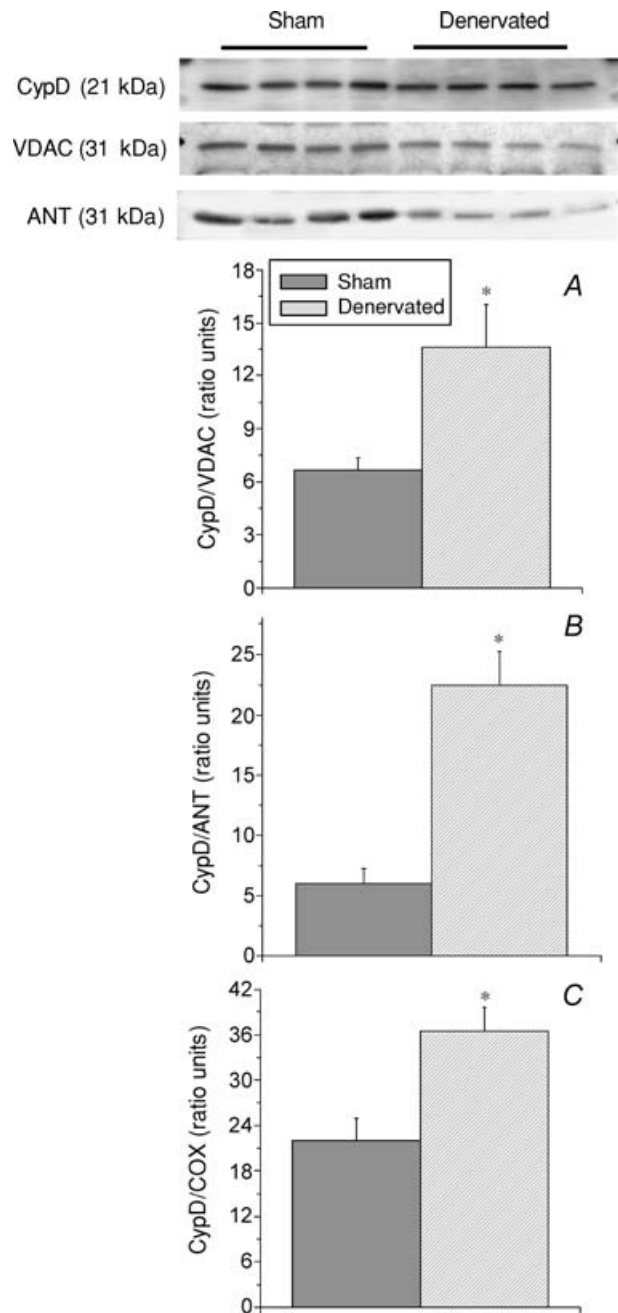


Figure 5. Cyclophilin-D, VDAC and ANT content of mitochondrial fraction from sham and denervated animals
Representative immunoblots of cyclophilin D (CypD: 21 kDa), voltage dependent anion channel (VDAC: 31 kDa) and adenine nucleotide translocase (ANT: 31 kDa) in muscle mitochondrial fraction isolated from sham and denervated animals. Each line represents a single mitochondrial preparation in each group. Densitometric analysis for each protein represents the mean \pm S.E.M. of 4 preparations in each experimental group. A and B show CypD content expressed relative to that of VDAC and ANT, respectively. C shows CypD content relative to the activity of COX measured in the same samples. *Significantly different from sham ($P < 0.05$).

In this regard CypD, a matrix peptidyl-prolyl *cis-trans* isomerase, was recently shown to play an important role in sensitizing the pore to Ca^{2+} (Baines *et al.* 2005; Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005). Indeed, in mitochondria from *Ppif*^{-/-} mice, which are devoid of CypD, the amount of Ca^{2+} required to trigger PTP opening is increased severalfold (Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005). In addition, the inhibitory effect of CsA is completely abolished in these mice, which indicates that CypD expression is the key factor that confers sensitivity to CsA (Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005). Consistent with these data, Baines *et al.* (2005) also reported that in mice over-expressing CypD in a cardiac-restricted manner, PTP opening occurred more readily and that CsA could prevent this effect. Our observations that CsA was selectively more potent in mitochondria from denervated muscle and was able to restore CRC to the level observed in sham-operated muscle therefore suggest a role for CypD in increasing the vulnerability to PTP opening following denervation. The fact that CypD was over expressed by 2- to 3-fold relative to all mitochondrial protein markers measured following denervation is consistent with this idea. To our knowledge, this is the first study to suggest a link between changes in CypD expression and PTP dysregulation in a non-transgenic model of disease.

Conclusion

In conclusion, the results reported in the present study indicate that conditions that substantially increase the vulnerability of mitochondria to undergo permeability transition are observed 21 days after denervation. This includes a significant increase in mitochondrial and whole muscle Ca^{2+} content and an increase in the expression of CypD, which further predispose the Ca^{2+} overloaded mitochondria to permeability transition. This observation is of significance as the occurrence of PTP opening in some mitochondria *in vivo* may partly account for the activation of mitochondria-mediated cell death that is known to occur in the process of denervation atrophy (Tews & Goebel, 1996, 1997a,b; Tews, 2002; Siu & Alway, 2005).

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